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AD-A233 372

CHARACTERIZATION OF BACTERIA FOUND IN METAL-WORKING FLUIDS AND THE WASTE TREATMENT SYSTEM INVOLVED IN DEGRADATION OF WASTE WATER

by

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THESIS

HQ, MILPERCEN

Submitted to the Graduate School

of Wayne State University

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

MASTERS OF SCIENCE

1991

20030207002

MAJOR: BIOLOGICAL SCIENCES
(Microbiology)

Approved by

Advisor date

91 3 14 021

DEDICATION

To my parents, Robert and Frances Cornwell. I would like to take this opportunity to thank you for everything that you have done for me.

ACKNOWLEDGMENT

I wish to express my gratitude to my advisor, Dr. Harold W. Rossmoore, whose continuous support has helped me accomplish my goal. Dr. Rossmoore is a very knowledgeable and caring man. I would like to thank him for all his help.

I would also like to thank the U.S. Army for selecting me to attend graduate school under the fully funded advanced civilian schooling program. Without this selection I would not have attended graduate school when I did. In addition, I would like to thank the members of my committee, Dr. J. M. Jay and Dr. K. C. Chen.

I would like to take this opportunity to extend a very special thanks to Helen Douglas and Veronica Riha for their friendship, technical assistance and encouragement during my research period.

FOREWORD

My main purpose for undertaking this study was to learn all I could in microbiology that would be beneficial to the U.S. Army. I was sent to Graduate School by the U.S. Army to attain a Masters in the field of microbiology. During my first semester at Wayne State University there were not many courses offered in microbiology and the only way I could think of to learn a considerable amount about microbiology was by undertaking a research project. At that time the Army was also interested in the degradation of oils and as a result of this I started this project; looking at the biodeterioration and biodegradation of metal working fluids. This project has helped me fulfill the requirements that I set upon myself; that of learning all I could in the field of microbiology with respect to biodeterioration and biodegradation of metal working fluids. This study and other laboratory work has introduced me to different techniques, equipment, and media that I would not have been exposed to if I had opted to undertake the Plan C Masters.

This thesis is written primarily for two audiences: the U.S. Army and Wayne State University. Thus, it must be remembered that persons not intimately involved in this field have a vested interest in my work.

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INTRODUCTION

The work for this Masters Thesis involved the study of microorganisms in Metal Working Fluids (MWF) and the fluidized bed reactors involved in their disposal. It was of interest in determining what bacteria survive in MWF under different treatments, both chemical and biological, and during the waste treatment process. These studies focused on bacteria isolated from MWF and from different locations in the waste treatment system. Also looked was the survival of bacteria, under laboratory conditions, after changes in their environment. The environmental changes included: changes in pH, biocide concentration (biocides used were the same ones used in the industrial plant), and carbon source. All samples used for this study were collected from a General Motors plant (Delco Moraine New Departure Hyatt (NDH) plant) in Sandusky, Ohio.

Because of the possibility of future assignment in this field (biodeterioration and/or degradation of oils) in the Army, an understand of what was occurring in these environments was needed by the author. From this research with MWF, results from these changes in the environment could be of interest to the Army. This study also helped one to understand the effects that changes in the environment had on the survival of bacteria. The areas of particular importance to this study were metal working fluids, biocides and the fluidized bed reactors used.

Metal Working Fluids

Metal working fluids are used in many industries today. Some types of metal working or processing methods that use MWF are [3]:

1.) machining and grinding

2.) stamping, blanking, drawing, and spinning

3.) molding

4.) rolling.

The primary function of the MWF is to increase the efficiency of these and other industrial operations. The MWF is employed for different functions depending upon the type of machine operation to which it is applied. The two basic functions which any MWF must perform are [24]:

1.) To remove heat from the cutting tool, work piece and chips (i.e. to cool).

2.) To reduce the friction between the flowing chips and the cutting tool, thus reducing the heat generated in cutting.

The major benefit from these functions is to improve tool life. There are also other functions that the MWF must perform to increase the efficiency of operation. These include [5]:

1.) provide rust/corrosion protection

2.) lubricate exposed machine tool parts

3.) reduce distortion through cooling

4.) allow for increased tool speeds

5.) wash away chips from the work piece/tool interface.

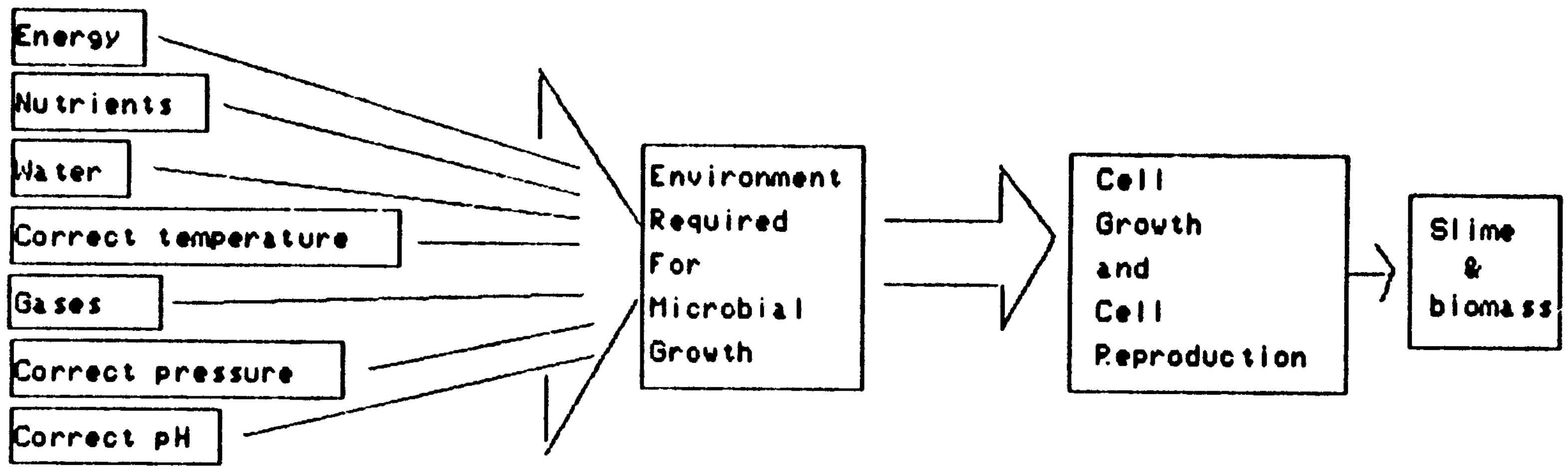
The type of MWF examined in this study was a water based MWF. With the addition of water to metalworking operations it has created a more than favorable environment for a variety of microorganisms. Since MWF is such an excellent environment for microbial growth, industries working with MWF are concerned about contamination. The massive amount of growth of microorganisms in MWF can cause adverse effects to the industrial operations. Some of the common problems due to microbial contamination are listed in Table 1 [20].

TABLE 1: PROBLEMS CAUSED BY MICROBIAL CONTAMINATION

- odor development
- decrease in pH
- changes in emulsion
- increase in corrosion rates
- changes in coolant chemistry
- decreased tool life
- surface-finish blemishes
- clogged filters, screens & lines
- increase work piece rejection rates

For growth of microorganisms in any environment, certain physiological and nutritional requirements must be met. Figure 1, shows the requirements for microbial growth [7]. If any one of these factors is lacking then cell growth stops. MWF becomes contaminated with microorganisms because the fluid contains all the required nutrients needed to suppo··t microbial growth. Some of the nutrients found in MWF are listed in Table 2 [20]. The mineral oil base

FIGURE 1: Physical and Nutritional Requirements foe the Growth of Microorganisms in MWF.



stocks, glycols, fatty acid soaps, amines, and other constituents of MWF provide all of the essential nutrients required for microbial growth. The other requirements for microbial growth: pH, temperature, pressure, and gases, are met through the normal operation and maintenance of the MWF.

TABLE 2: NUTRIENTS IN METAL WORKING FLUIDS

Organic	Inorganic
	cation:
- mineral waxes	- iron
- fatty oil	- calcium
- fatty acid soaps	- sodium
- synthetic esters	- magnesium
- phosphate esters	- manganese
- amines	
	anions:
	- sulfate
	- chloride
	- phosphate

Large populations of microorganisms are readily detected because of their effects on the fluid or system (see Table 1). Unfortunately, by the time these effects are noticed, it may already be too late to rescue the system and prevent further damage. In addition, since microbes are so small and cannot be seen by the naked eye, the biodeterioration problems that they cause are often not recognized until it is too late [8]. The growth of the bacteria in the MWF can be controlled by the addition of preservatives. The preservatives that are of interest to this study are biocides.

Biocides

Biocides are added to MWF to destroy/inhibit microbial cells. Some of the ways that biocides react (mode of action) with the microorganisms are described below. Two of the biocides used in this study, A and B, are considered formaldehyde (FA) releasers. This was demonstrated by the extensive work of Rossmoore et al [22, 23]. It was found that the release of FA from the biocide was essential for its mode of action on the microbial cells. When FA was released, it reacted with essential nucleophiles (SH groups and amines) in the biological environment and then reacted with the cells causing loss of viability. Biocide C also reacts in a similar manner with the nucleophiles.

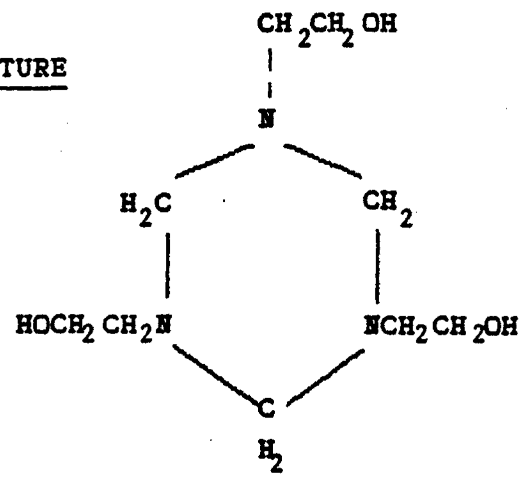
Another probable mode of action involves the biocide making contact with the cell envelope and thus gaining cell entry. Some entry can be contributed to the oil-water portion of the cell envelope, thus allowing the biocide to penetrate the lipophilic cell envelope. Some components of the biocides also have structures similar to the essential nutrients and metabolites; it is therefore possible to envision a form of competition for some of them [23]. As a result of the action of the biocide on the microbial cells, the cells may stop cell growth and reproduction.

The biocides in this study were used in the MWF in the General Motor Plant and these are outlined below.

1. <u>Hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine</u>

   ($C_9H_{21}N_3O_3$)

STRUCTURE



EFFICACY: This biocide is an effective antimicrobial agent that can be used to inhibit the growth of bacteria in aqueous-based metal working fluids. It is effective against gram-positive and gram-negative bacteria. It was tested for efficacy at the recommended concentration of 1500 ppm (parts per million). The physical properties of this biocide are listed in Table 3 [13-15] and it is referred to as "Biocide A".

TABLE 3: TYPICAL PHYSICAL PROPERTIES OF HEXAHYDRO-1,3,5-TRIS(2-HYDROXYETHYL)-S-TRIAZINE

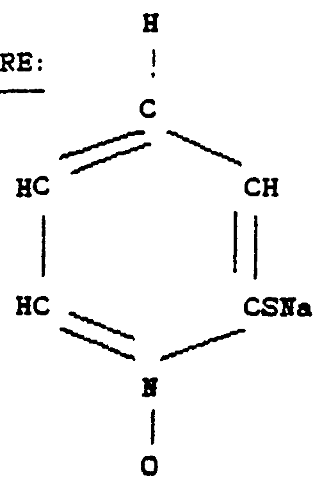
- active ingredient (%)	78.5
- inert ingredient (%)	21.5
- color	amber
- odor	faint amine

2. 1-Hydroxy-2(1H)-Pyridinethion, Sodium salt and Hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine

This is a mixture of two other known biocides. The two components are:

a. <u>Sodium 2-pyridinethiol-1-oxide (C_5H_4NOSNa)</u>

<u>STRUCTURE:</u>



b. <u>Hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine</u> ($C_9H_{21}N_3O_3$). See to Biocide A for structure.

<u>EFFICACY</u>: This biocide utilizes a potentially synergistic combination of an antifungal agent and an antibacterial agent. It provides pronounced growth inhibiting activity against gram-positive and gram-negative bacteria, yeasts, and molds. The antifungal agent is sodium-2-pyridinethiol- 1-oxide and the antibacterial agent is hexahydro-1,3,5-tris (2-hydroxyethyl)-2-triazine. This biocide eliminates the need for adding two antimicrobial agents. It was tested for efficacy at the recommended concentration of 1000 ppm. Table 4 lists some of the typical properties of this biocide [16-18] and it is referred to as "Biocide B".

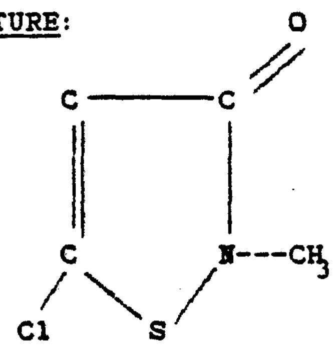
TABLE 4: TYPICAL PHYSICAL PROPERTIES OF "BIOCIDE B"	
Active Ingredient (%)	70
Color	amber

3. This biocide is a mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one

a. 5-chloro-2-methyl-4-isothiazolin-3-one

(C_4H_4NOSCl)

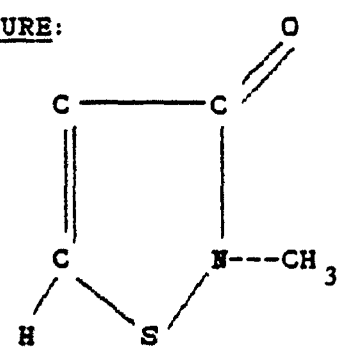
STRUCTURE:



b. 2-methyl-4-isothiazolin-3-one

(C_4H_5NOS)

STRUCTURE:



EFFICACY: This biocide is used to inhibit the growth of bacteria and fungal contaminants in aqueous dilutions of emulsifiable synthetic and semi-synthetic MWF. Some of the physical and chemical properties of this biocide are listed in Table 5 [21]. The recommended level of use as established for EPA regulation is 100 ppm. This biocide is referred to as "Biocide C".

TABLE 5: PHYSICAL AND CHEMICAL PROPERTIES OF "BIOCIDE C"

Active Ingredients	
- 5-chloro-2-methyl -4-isothiazolin-3-one	8.6 % min.
- 2-methyl-4-isothiazolin -3-one	2.6 % min.
Appearance	pale yellow
Odor	mild, aromatic

Because of the differences in location of their isolation, it was of interest to see if any of the microorganisms showed a difference in their minimal inhibitory concentration (MIC). The MIC is the concentration of an antimicrobial agent necessary to inhibit the growth of a particular strain of microorganism [1]. This test determines the concentration of an antimicrobial agent that is effective in preventing growth of the organisms and gives some indication of the minimal dosage that should be effective in controlling the microbes. It is important to realize that in a given species biological sensitivity can vary in its MIC by a factor of 2-5, i.e. the differences in their intrinsic resistance [9]. Many authors have reported the resistance of microorganisms to toxic chemicals and the ways in which microorganisms acquire their resistance [6, 9, 10, 25, 26, 27]. It was desirable to see if the bacteria showed any differences in their MIC based on the location of the isolate. In addition, it was of interest to determine if these MIC differences were also evident in a medium stressing hydrocarbon utilization.

Waste Treatment System

The biological waste system used to biodegrade waste MWF was the OXITRON fluidized bed reactor. Waste MWF first passed through a physical-chemical treatment before it reached the fluidized bed reactor. Figure 2, shows a detailed schematic of the waste water process at Delco Moraine NDH Plant. This system is divided into a physical-chemical and a biological section.

The physical-chemical section involves seven steps from the primary clarifier to sand filter # 1. The primary clarifier is responsible for the removal of free oil and any solid material from the waste. The waste then goes into one of three holding tanks where it is allowed to sit for a period of time, allowing settling of more oil and its removal by skimming from the top. (The waste sits in one of the holding tanks while the other two are being filled and emptied of waste MWF.) After passing the holding tank, the waste water goes through a series of chemical treatments that further break down the waste material. In mix tank # 1, chemicals are added to lower the pH to approximately 4.8. This is done to split emulsion and further break down the

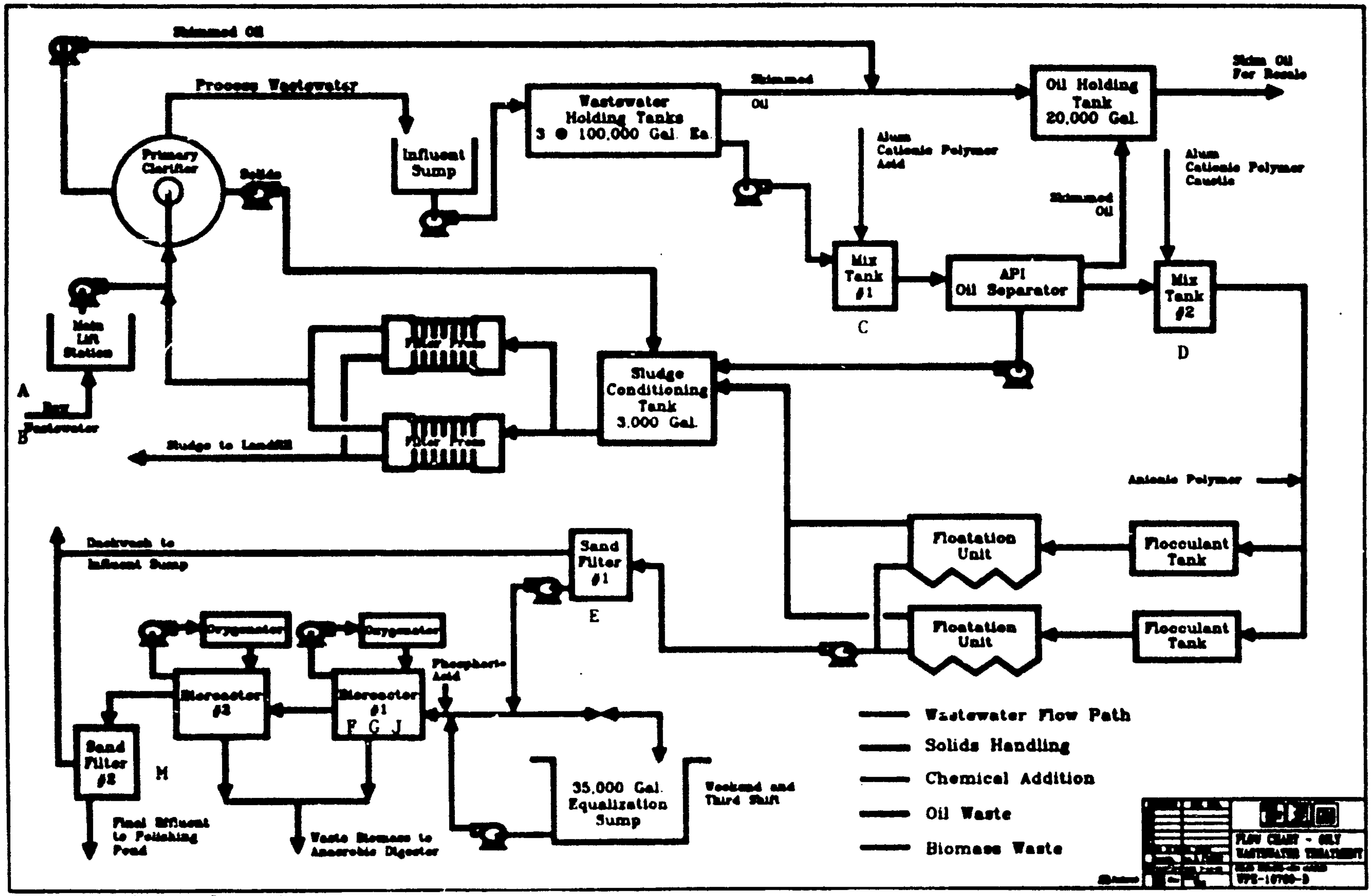


Figure 2: Schematics of Waste Treatment System

chemical components in the waste MWF. The waste then goes through another clarifier, the API oil separator, where more free oil is removed. Next is mix tank # 2, where more chemicals are added and this time the effect is to raise the pH to approximately 9.2. Finally, the waste material goes through the flotation units to remove any more solids that are brought to the top by dissolved air. From here the waste water material enters the biological portion of the system.

The Delco Moraine NDH system, where samples were collected, was one of the first General Motors Plant to install a full scale aerobic fluidized bed OXITRON system [12]. The OXITRON is a commercial embodiment of the aerobic biological fluidized bed process configuration system developed by Dori-Oliver, Inc. Figure 3 shows the OXITRON aerobic fluidized bed process schematics [12]. In this system, the waste water is passed upward through a rectangular or circular reactor containing a bed of sand (or granular activated carbon media may be used) at a velocity sufficient to expand the bed, resulting in a fluidized state. In this system, the combination of liquid and solid particles display what Leva [11] called particulate fluidization. The bed expands smoothly with none of the violent bubbling and particle motion characteristic of gas/solid fluidization, known as aggregative fluidization [11]. Once fluidized, the media particles provide a vast surface area for biological growth, leading to the

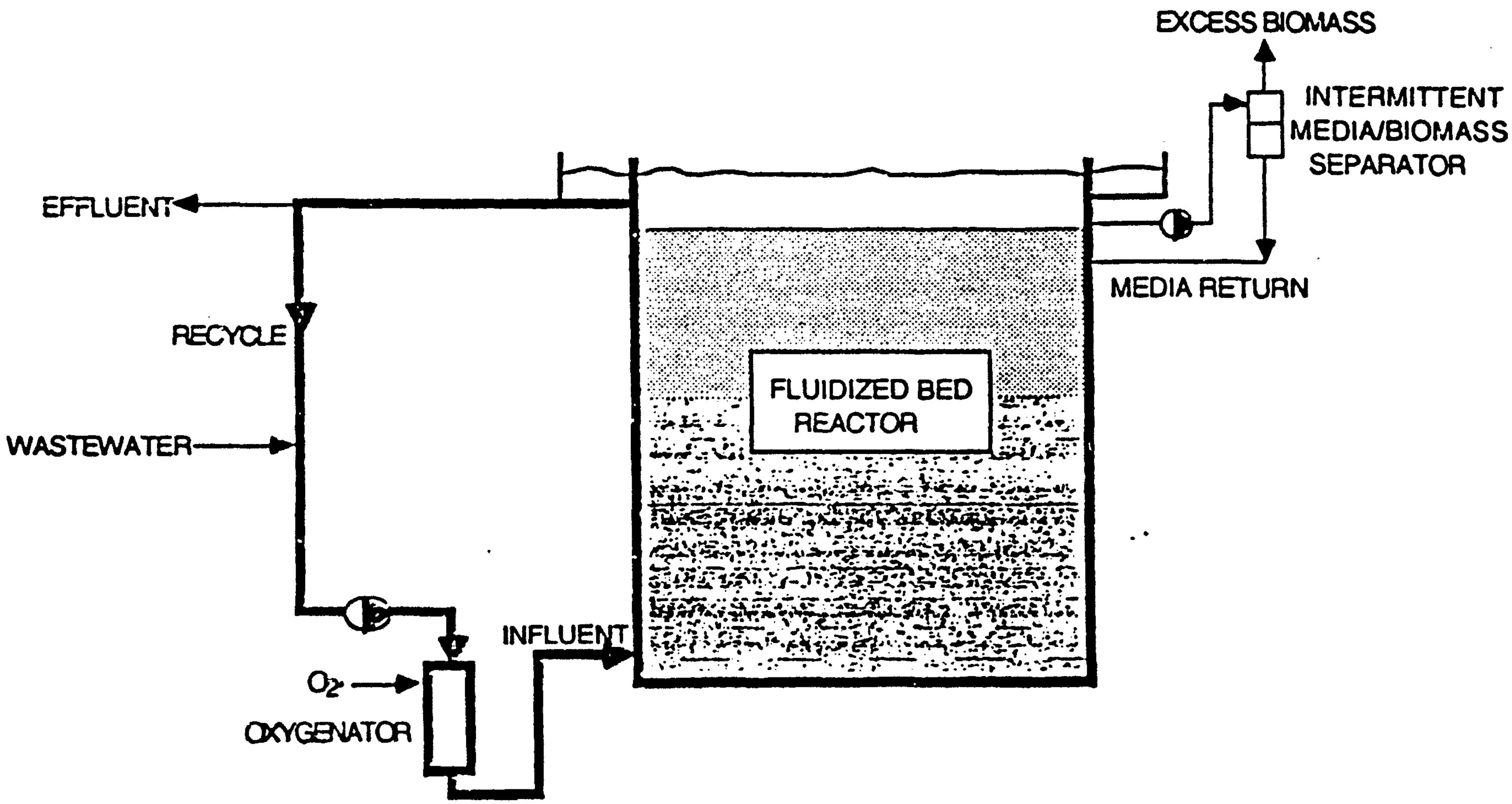


Figure 3: OXITRON Aerobic Fluidized Bed Process Schematic
From Biological Fluidized Beds for Water and Waste Water Treatment

development of a biomass concentration approximately five to ten times greater than that normally maintained in conventional bioreactors. As a result of the biomass concentration on the media particles, they tend to become stratified in the bioreactor. It was shown that vertical density gradient could be determined as a result of the stratification [11]. The particles at the top of the fluidized bed had a greater biomass concentration than those at the bottom. Degradation of the waste water is accomplished by the microorganisms found in the biomass. Microbial degradation of complex organic compounds requires the maintenance of a long solid retention time in the biotreatment reactor, corresponding to a slow net growth rate of the microbial population. The high reactor biomass concentration allows achievement of a long solid retention time at a short liquid hydraulic retention time [28]. The use of dissolved oxygen in the influent stream satisfies the oxygen requirements.

The Delco Moraine NDH system was installed in late 1985 and early 1986 and consists of two fluidized bed reactors. The combine physical-chemical and biological process is designed to treat up to 544 m^3/day of industrial waste water [28]. The first reactor in this system is for carbonaceous oxidation and the second reactor is for nitrification. The effluent from the oily waste water treatment system is deficient in phosphorus; therefore, phosphorus is added to the first reactor in the form of phosphoric acid. In addition, sodium hydroxide is added to buffer the carbon dioxide produced during the carbonaceous oxidation and also to buffer the hydrogen ions produced during the nitrification process.

The samples collected from the waste water treatment system are listed in Table 6. Refer to Figure 2 for location on the schematics chart. The samples sites were chosen based on the differences in the chemical treatment in the system.

TABLE 6: LOCATION OF THE SAMPLES COLLECTED

Identifier	Location
A	MVF from the north side
B	MVF from south side
C	Mix tank # 1
D	Mix tank # 2
E	Influent into sand filter # 1
F	Reactor 1 - free flowing liquid
G	Reactor 1 sand at 10 1/2 ft
H	10 1/2 ft sand gently shaken
I	10 1/2 ft sand sonic oscillation
J	Reactor 1 sand at 20 ft
K	20 ft sand gently shaken
L	20 ft sand sonic oscillation
M	Effluent - going to pond

Objectives

The main objective of this project was to characterize bacteria found in various locations of the waste water treatment and to determine whether organisms of the same species showed differences in response to the changes in their environment based on the location from which they were isolated.

METHODS AND MATERIALS

All tests performed in this research were run two or more times to reinforce the validity of findings.

Microbiological Evaluation

Samples were collected from the General Motors Plant in plastic screwcap jars on 9/28/90 and 10/1/90. The samples were put on ice and transported to Wayne State University where microbiological evaluation was carried out. The bacterial count in the sample was determined by serial dilution on Plate Count Agar (PCA, Difco Laboratories, Detroit,MI) for all locations except location H, I,K, and L. The samples from these location involved sand. Removal of bacteria from the sand was achieved by putting 1 gram of sand into a Zip-lock bag with 9 ml of sterile water. Removal was done by gently shaking the bag and another was also put in the sonic oscillator for 1 minute and subsequently diluted and plated. The plates were incubated at 33 C for 48 hours.

Media and Culture Conditions

All cultures were maintained on tryptic soy agar (TSA, BBL, Detroit MI). The cultures were kept fresh by being transferred to new TSA every ten days. Twenty-four-hour-old cultures grown in tryptic soy broth (TSB, Difco Laboratory, Detroit, MI) were used for determining the pH range, MIC concentration and growth in the mineral salts based medium.

The bacterial population in the broth at 24 hour was approximately 10^9 bacteria/ml. Thus, the microbial population inoculum used in these tests was 0.1 ml of a 24-hour culture yielding a final level in the system of 10^6 CFU/ml.

Isolation & Identification of Organisms

Various colony types were cultured onto TSA. Colonies were gram stained for purity before being restreaked, to insure a pure culture stock. The bacterial isolates were all gram-negative; therefore, it was possible to use the PASCO I.D Tri-Panels for Gram-negatives to identify the microorganisms (see Appendix for instructions on the use of the PASCO panels).

Determining pH Range of Organisms

The pH sensitivity tests were done with both TSA and TSB. With the TSA, pH was adjusted to a range of 4-8 and with the TSB, the pH was adjusted to 3.5 to 9. Both media were sterilized before the pH was adjusted with HCl or NaOH. With the TSA, pH was tested with all the microorganisms to get the general pH range for the microorganism. The pH range was checked again, using TSB, to those microorganisms listed in table 17 to see if the results would be the same as those for TSA. It was of interest to see how acidic the media could be without affecting the survival of the bacteria.

Another test with pH variation was done on the sand from the bioreactor. Here, one wanted to see if the organisms still attached to the sand could survive a lower pH than those that were freed. The question asked of the author was "What would the pH have to be lowered to inhibit the growth of the bacteria still attached to the sand (biofilm) ?"

To answer this question, a gram of sand from the bioreactor was placed into a 250 ml flask containing 100 ml sterile TSB. The pH of this medium then adjust to the following pH: 4.5, 5.0, 5.5, 6 and 7. The flasks were incubated at 33 C for 48 hours. Positive results or growth was indicated by turbidity of the medium.

Determining Biocide MIC

Based on the location of the bacteria isolated, it was also of interest to see the differences in MIC. These tests were performed using 0.1 ml of 24 hour old cultures grown in TSB and added to 10 ml of TSB. The tests were performed in standard test tubes set up for serial dilution. The amount of biocide added to the first test tube of TSB was 1/10 the published application concentration, which for biocide A was 1500 ppm (0.15 %) of biocide in solution, biocide B was 1000 ppm (0.1 %) of biocide in solution, and biocide C was 100 ppm (0.01 %) of biocide in solution. The dilutions used are listed in Table 7.

TABLE 7: Dilutions used in Biocide Testing (ppm)						
Biocide A	100	50	25	12.5	6.25	3.125
Biocide B	100	50	25	12.5	6.25	3.125
Biocide C	10	5	2.5	1.25	.625	.3125

After these tests, a more definite end point for the MIC was determined. The dilutions used for these tests are listed in Table 8. For biocide A, a too low of a biocide concentration was used and the results were all positive.

TABLE 8: Dilutions of Biocides for End Point						
Biocide A	10	7.5	5	3.75	2.5	1.875
Biocide B	100	75	50	37.5	25	18.75
Biocide C	10	7.5	5	3.75	2.5	1.875

Determining Growth of Organisms in Mineral Salts base Medium

The final experiment performed was to determine which microorganisms would grow in a mineral base medium described by Palleroni and Doudoroff [19]. This medium consisted of M/30 NaK phosphate buffer pH 6.8, NH_4Cl (0.1%), $MgSO_4*7H_2O$ (0.05%), ferric ammonium citrate (0.005%), $CaCl_2$ (0.0005%) and a carbon source (0.1%). The carbon source used in this case was the hydrocarbon n-hexadecane. The media was prepared and sterilized after which the hydrocarbon was added. The microorganisms used in this test are listed in

Table 20. The test was done in 100 ml flasks with 50 ml media and a 0.1 ml of 24 hour old culture sample. These were then put into a G24 environmental incubator shaker (New Brunswick Scientific), set at 28 C and 200 RPM. These samples were looked at daily for evidence of turbidity indicating growth. Those with growth were used for another test to see if they would still grow in this medium with the addition of Biocide C. Biocide C was selected because of its lower effective dose. The amount of Biocide C added was 2.5 ml of 100 ppm stock solution thus making the final biocide concentration 5 ppm. This amount was used because most of the bacteria tested survived at this concentration. The rationale behind this is that it was known that the microorganisms would grow in each test separately but could they survive in the mineral base medium plus the hydrocarbon and the biocide.

RESULTS

Bacterial Level

The results for the CFU/ml based on location are presented in Table 9. The results for the CFU/ml were obtained by selecting and counting the plates with 30 to 300 colonies. The Quebec counter was used to make the count. The formula used for determining CFU/ml was:

$$\frac{CFU}{ml} = \frac{\text{(average number of colonies/plate)}}{\text{(dilution plated)(volume plated in ml)}}$$

Locations A through D have two sets of results because in the first set, it was believed that the agar was poured to hot based on the low count values. As outlined in Table 9, there was a slight increase in the second set of results compared to the first set. The results for A through D were much lower than the rest of the results because of their location. Location A through D are for MWF and for those collected from the physical-chemical portion of the waste treatment system.

For location D, the crowded plate method was used to determine the count because there was no growth on any of the higher dilution plates. The probable reason being, that the agar was poured too hot. The crowded plate method was done by counting the colonies in five square areas. These counts were then averaged and multiplied by sixty-four (area of the petri dish). A third count was not performed because the sample was five days old and the count would not have been as accurate.

TABLE 9: Results for Bacterial Colony Forming Units per Location

Location	CFU/ml	Average
A	1st 89×10^{3}	
	118×10^{3}	1.0×10^{5}
	2nd 191×10^{3}	
	122×10^{3}	1.6×10^{5}
B	1st 14×10^{4}	
	32×10^{4}	3.2×10^{5}
	2nd 165×10^{4}	
	169×10^{4}	1.7×10^{6}
C	1st 153×10^{2}	
	109×10^{2}	1.3×10^{4}
	2nd 251×10^{2}	
	262×10^{2}	2.6×10^{4}
D	1st 36×10^{2}	
	39×10^{2}	3.8×10^{3}
	2nd 1433×10^{2}	
	1267×10^{2}	1.4×10^{5} done by crowded plate method

TABLE 9 (cont.): Results for Bacterial Colony Forming Units per Location

Location	CFU/ml	Average
E	65×10^{5} 63×10^{5}	6.4×10^{6}
F	108×10^{5} 159×10^{5}	1.3×10^{7}
H CFU/gm sand	51×10^{6} 243×10^{5}	3.8×10^{7}
I CFU/gm sand	296×10^{7} TNTC	3.0×10^{9}
K CFU/gm sand	98×10^{5} 161×10^{5}	1.3×10^{7}
L CFU/gm sand	44×10^{7} 101×10^{7}	7.3×10^{8}
M	202×10^{3} 97×10^{3}	1.5×10^{5}

G - Did not do because H & I are the results using different removal techniques.

J - Did not do because K & L are the results using different removal techniques.

The results between the gently shaking and sonic removal in H & I and K & L were what one expected. The removal by the sonic bath removed more bacteria from the sand than by just gently shaking the sample. The first thoughts that one would think about the results for location M was that the count was too low. This was the sample that was going to the pond and microorganisms found here were the free living bacteria from the bioreactors. These were the bacteria that Andrews described as being individual cells that were so small and light that they were washed out of the bioreactors [2]. The overall results were what one expected based on the locations that they were collected.

Identification of Microorganisms

The results for the identification by the PASCO ID TRI Panels are given in Table 10. The results are listed by biotype number first and then by genus and specie name if the biotype number matched up in the PASCO I.D. book. From the results, it showed that it was not always possible to isolate specific microorganism in each area. However, *Acinetobacter lwoffi* was found in most of the locations and it was this microorganism that was tested.

There are two possibilities for the failure cited above. First, all the microorganisms possible from each location were not isolated. If one had been selective, there might have been greater success in the isolation of microorganisms common to each location. The second reason for not getting

TABLE 10: The Results of the Identification of Microorganisms by the PASCO ID TRI-Panels

Location Bacteria #	A	B	C	D	E	F	H
1	000004010	000004401 Aci. lwoffi	075430302	000000100 Aci. lwoffi	000000000 Aci. lwoffi	000000000 Aci. lwoffi	200700200
2	000004012 Pseud. spp.	000044004	265740613	000000401	000000000 Aci. lwoffi	000000020 Moraxella	
3	000040037	200746216	377700000	000004000 Aci. lwoffi	000000000 Aci. lwoffi	000000020 Moraxella	
4	240044012	240746026		000004012 Pseud. spp.	000000040 Aci. woffi	000000040 Aci. lwoffi	
5	352005207	241006000		200000020	000000120		
6	366005005	252001401		200004020	100000000		
7	366400400	377700401		200004020	360744000		
8	4 weren't ID	377700000	3 weren't ID			1 wasn't ID	2 weren't ID
9		770007412					

NOTE: The names below the biotype number are only listed for the ones with a sure match in the ID book. I also listed the number of microorganisms that I could not identify because they would not grow in the positive control well of the PASCO ID Tri-panels.

TABLE 10: Continuation of Identification of Microorganisms

Location Bacteria #	I	K	L	M
1	000000002 Aci.lwoffi	000000002 Aci.lwoffi	000740020	000000000 Aci. lwoffi
2	000000020 Moraxella	040000620	040004000	000000000 Aci. lwoffi
3	000017005	240000042	240540620	000000000 Aci. lwoffi
4	000075441			710000002
5	200300020			
6	377700002	1 wasn't ID	1 wasn't ID	1 wasn't ID
7	1 wasn't ID			

See note on first page of table 10.

a common microorganisms in each location might be due to the fact that some microorganism were only present in low numbers in that specific location. As it was stated earlier, Acinetobacter lwoffi was found in all locations except in Location C (mix tank # 1) where the pH is lowered to 4.8 due to the addition of chemical. It is possible that this organisms might not of survived this treatment of Location C. From here on out, the various tests were done with mostly Acinetobacter lwoffi and a few other bacteria.

Biocide MIC Results

For the biocide testing ten isolates were selected that keyed to Acinetobacter lwoffi from different locations (two of the samples used looked similar to Acinetobacter lwoffi based on colony characteristics) and challenged them with the three biocides. Table 11 gives the ten bacteria used for these tests by biotype number and their location. The results for these ten bacteria are presented in Tables 12 through 16. The interpretation of each table is given below its respective table. The MIC for each biocide is much less than the recommended level for industrial application.

MIC tests with Biocides B and C on different micro-organisms from various locations to see if their MIC was essentially the same as that for Acinetobacter lwoffi. Table 17 gives the bacteria used for these tests by biotype and location. Tables 18 and 19 give the results of these tests, with interpretations below each table.

TABLE 11: Bacteria used in the MIC Testing

Bacteria number	Biotype number	Location	Bacteria name
1		H	resembled Aci. lwoffi
2	000000000	F	Aci. lwoffi
3	000000000	E	Aci. lwoffi
4	000000000	E	Aci. lwoffi
5	000000000	M	Aci. lwoffi
6	000000002	I	Aci. lwoffi
7	000000401	D	Aci. lwoffi
8	000004401	B	Aci. lwoffi
9		A	resembled Aci. lwoffi
10	000000000	F	Aci. lwoffi

Bacteria 1 & 9 did not grow in the positive control well of the PASCO ID Tri-panels.

TABLE 12: Results of MIC Testing with "Biocide A"

Bacteria / [] ppm	1	2	3	4	5	6	7	8	9	10
3.125	++	++	++	++	++	--	++	++	++	++
6.25	++	++	++	++	++	--	++	++	++	++
12.5	++	++	++	++	++	--	++	++	++	++
25.0	++	++	++	++	++	--	++	++	++	++
50.0	++	++	++	++	++	--	++	++	++	++
100.0	++	--	+-	+-	--	--	--	--	--	--

+ means positive for turbidity
- means negative for turbidity

INTERPRETATION: The MIC for these microogranisms was greater than 50.0 ppm, Bacteria # 6 did not grow in the lowest concentration and it was assumed that the beginning inoculation was not viable. For the endpoint MIC, the tests were run using to low of a concentration and the results were all positive so the results were not included. This biocide was the last one tested so not as many microorganisms were tested with it.

TABLE 13: Results of MIC Testing with "Biocide B"

Bacteria / [] ppm	1	2	3	4	5	6	7	8	9	10
3.125	++	++	++	++	++	++	++	++	++	++
6.25	++	++	++	++	++	++	++	++	++	++
12.5	++	++	++	++	++	++	++	++	++	++
25.0	++	++	++	++	++	--	--	++	++	++
50.0	++	--	++	++	--	--	--	--	++	--
100.0	--	--	--	--	--	--	--	--	--	--

+ means positive for turbidity
- means negative for turbidity

INTERPRETATION: The results from this test showed that the MIC for this biocide in most bacteria tested here was greater than 25 ppm. For the results of a finer MIC endpoint refer to table 14. This biocide was also tested with other bacteria from various locations and the results are given in table 18.

TABLE 14: Results of MIC Testing with "Biocide C"

Bacteria / [] ppm	1	2	3	4	5	6	7	8	9	10
0.3125	++	++	++	++	++	++	++	++	++	++
0.625	++	++	++	++	++	++	++	++	++	++
1.25	++	++	++	++	++	++	++	++	++	++
2.50	++	++	++	++	++	++	+-	++	++	++
5.0	++	++	++	++	+-	++	--	++	++	++
10.0	--	+-	+-	+-	--	--	--	--	-	--

+ means positive for turbidity

- means negative for turbidity

INTERPRETATION: See Table 15 for the a finer range for the MIC endpoint. In these results it shows that the MIC is greater than 5.0 ppm in most of the bacteria. This biocide was used on other bacteria from the different locations, the results for these bacteria are shown in Table 19.

<u>TABLE 15: Results of MIC Endpoint with "Biocide B"</u>

Bacteria --------- [] ppm	1	2	3	4	5	6	7	8	9	10
18.75	++	++	++	++	++	--	++	++	++	++
25.0	++	++	++	++	++	--	++	++	++	++
37.5	--	--	++	++	--	--	++	--	--	--
50.0	--	--	--	--	--	--	+-	--	--	--
75.0	--	--	--	--	--	--	--	--	--	--
100.0	--	--	--	--	--	--	--	--	--	--

+ means positive for turbidity

- means negative for turbidity

<u>INTERPRETATION</u>: Bacteria # 6 did not grow in the lowest concentration in this testing but it grow in the previous testing with this biocide. It is assumed that this bacteria was not viable when the tests was started. The final MIC endpoints for the rest of the bacteria was greater than 25.0 ppm. See the table for the exact endpoint for each bacteria.

TABLE 16: Results of MIC Endpoint with "Biocide C"

Bacteria / [] ppm	1	2	3	4	5	6	7	8	9	10
1.875	++	++	++	++	++	--	++	++	++	++
2.5	++	++	++	++	++	--	++	++	++	++
3.75	++	++	++	++	++	--	++	++	++	++
5.0	++	++	++	++	++	--	--	++	+-	++
7.5	+-	+-	++	++	+-	--	-+	--	--	+-
10.0	--	+-	+-	++	+-	--	--	--	--	+-

+ means positive for turbidity
- means negative for turbidity

INTERPRETATION: Bacteria # 6 did not show any growth in this test but it showed growth in the first testing with "Biocide C", so it is assumed that this bacteria was not viable to begin with. The rest of the bacteria show a final MIC endpoint greater than 5.0 ppm except bacteria # 7 and 9. See the results for accurate endpoint.

TABLE 17: Bacteria used in MIC Testing for Table 18 and 19

Bacteria Number	Biotype Number	Location	Name
1	252001401	B	Aci. anitratus
2	240746026	B	
3	377700401	B	
4	200746216	B	
5	366400400	A	
6	240044012	A	
7	000040437	A	
8	352005207	A	
9	241006000	B	
10	000004010	A	
11	000004012	A	Pseud. spp.
12	no growth	A	
13	366005005	A	
14	000004012	D	Pseud. spp.
15	377700000	C	
16	no growth	A	
17	000000120	E	
18	040000620	K	
19	000740020	L	
20	000000022	I	Moraxella
21	040004000	L	

NOTE: The words "no growth" indicate that these bacteria did not grow in the control well of the PASCO system, therefore a biotype number could not be determined. The names of the microorganisms are only provided for the ones whose biotype number keyed to a name.

TABLE 18: Results of MIC Testing with "Biocide B" with other Isolated Microorganisms

[ ] ppm / bacteria	100	50	2.5	12.5	6.25	3.125
1	--	++	++	++	++	++
2	--	++	++	++	++	++
3	--	++	++	++	++	++
4	--	++	++	++	++	++
5	++	++	++	++	++	++
6	--	++	++	++	++	++
7	--	--	++	++	++	++
8	--	--	++	++	++	++
9	--	--	+-	++	++	++
10	--	++	++	++	++	++
11	--	++	++	++	++	++
12	--	--	--	++	++	++
13	--	--	++	++	++	++
14	--	++	++	++	++	++
15	--	++	++	++	++	++
16	--	--	--	++	++	++
17	--	--	++	++	++	++
18	--	--	--	++	++	++
19	--	--	--	++	++	++
20	--	--	++	++	++	++
21	--	--	+-	++	++	++

INTERPRETATION: As the reults indicated, most of the bacteria have a MIC greater than 25 ppm. There a'so was a variance between the microorganisms that might be a result due to the location that the bacteria were isolated from. Compared to the *Acinetobacter lwoffi*, some of these bacteria are more resistant to this biocide.

TABLE 19: Results of MIC Testing with "Biocide C" with other Isolated Microorganisms

[] ppm / bacteria	10	5	2.5	1.25	.62	.31
1	--	++	++	++	++	++
2	--	--	++	++	++	++
3	--	--	--	++	++	++
4	--	--	++	++	++	++
5	--	--	--	++	++	++
6	--	--	++	++	++	++
7	--	--	++	++	++	++
8	--	--	++	++	++	++
9	--	--	--	++	++	++
10	--	++	++	++	++	++
11	--	--	+-	++	++	++
12	--	+-	++	++	++	++
13	--	--	++	++	++	++
14	++	++	++	++	++	++
15	--	++	++	++	++	++
16	--	--	--	--	--	+-
17	--	--	--	++	++	++
18	--	--	--	--	--	++
19	--	--	--	--	++	++
20	--	--	--	--	++	++
21	--	--	--	--	++	++

INTERPERTATION: As the results indicates there is a variance in the data based upon the bacteria being tested. The results from the previous tests were using the same microorganisms but from different locations. The results shown here are different microorganisms and different locations. Compared to Acinetobacter lwoffi results, the bacteria here are more sensitive to this biocide.

PH Range Results

The pH tests were performed on all of the bacteria isolated on TSA. Bacteria listed in Table 17 were also tested in TSB to see if there was a differences based on the medium being a liquid or solid. The results from both tests showed that most of the bacteria survived a pH range of 6 to 8. Some of the Bacteria tested in TSB also grew at pH 9 but not any lower than pH 6.

The results of the pH tests done on the sand from the bioreactor showed that they could grow at a lower pH than the isolated bacteria. There was obvious growth, based on turbidity, in pH 7, 6, 5.5 and 5. There was survival in pH 4.5, determined by surface plating on TSA, not by obvious growth. The bacteria isolated from surface plating were also used in subsequent studies in mineral salts-hydrocarbon medium.

Results indicated that the organisms attached to the sand could survive a lower pH environment then the isolated microorganisms. The question asked "How much would you have to lower the pH to inhibit the growth of the organisms on the sand ?" From these initial tests, I would say that the pH of the bioreactor would have to be lowered to a pH below 4.5 to produce inhibition.

Mineral Base Medium Results

The microorganisms that were used in this test are listed in Table 20. Turbidity was used as a means of determining growth. The samples were examined at each day for obvious growth while they were in the shaker. The appearance of obvious growth, approximately 10^7 bacteria/ml, took approximately ten days. Table 20 also indicated which microorganisms showed obvious growth on the day they were taken from the shaker (day 11). These bacteria were then used for the mineral base medium plus the hydrocarbon and biocide test.

These results were evaluated each day for obvious growth and after ten days then was no apparent growth. After three weeks in the shaker, none of the samples showed apparent growth, so serial dilution were performed on PCA to see if the bacteria were surviving. The results of the bacterial load in this medium are given in Table 21.

TABLE 20: Bacteria used in the Mineral Base Medium Test

Biotype	Location	Name	Growth in Medium
000004401	B	Aci. lwoffi	yes
sand isolate	G	?	yes
sand isolate	G	?	yes
sand isolate	G	?	yes
sand isolate	G	?	yes
no growth	A	?	yes
000000401	D	Aci. lwoffi	yes
000004012	A	Pseud. spp.	yes
000000020	I	Moraxella	yes
no growth	A	?	yes
no growth	A	resembled Aci.	no
no growth	H	resembled Aci.	no
000000000	F	Aci. lwoffi	no
000000000	F	Aci. lwoffi	no
000000000	E	Aci. lwoffi	no
000000000	E	Aci. lwoffi	no
000000000	M	Aci. lwoffi	no

Note: Sand isolates refer to the ones that were isolated from the pH testing on the sand of the bioreactor. The pH was lowered to 4.5.

Table 21: Results of Mineral Base Medium and Biocide Bacterial Count

Biotype	Location	Growth
000004401	B	> 1000 CFU/ml
sand isolate	G	> 1000 CFU/ml
sand isolate	G	> 1000 CFU/ml
sand isolate	G	> 1000 CFU/ml
sand isolate	G	> 1000 CFU/ml
no growth	A	> 1000 CFU/ml
000000401	D	> 1000 CFU/ml
000004012	A	> 1000 CFU/ml
000000020	I	> 1000 CFU/ml
no growth	A	> 1000 CFU/ml

NOTE: When the samples were plated, the dilutions plated were from 10 to the 3rd to 10 to the 6th. The amount of growth on the 10 to the 3rd plates were less than 30 colonies and could not be counted. Thus the number of bacteria in the medium is given as less than a 1000 to indicate that there was survival but no growth. Since 0.1 ml of a 24 hour culture was put into 50 ml of the medium (10 to the 5th), the results indicates a decline in the microbial population. The reasons for the decline might be because the environment was to stressful for survival.

CONCLUSION

The results obtained from this project revealed many things about the microbial population in the MWF and the waste treatment systems. As expected the microbial load in the bioreactors is much higher than any place else in the system. The control of bacteria in the MWF by biocides is done to prevent the biodeterioration of the MWF and to prevent other contamination problems caused by the microorganisms (table 2). The level in the bioreactors is expected to be higher because it is in the bioreactors that the bacteria are responsible for the biodegradation of industrial waste water.

The bacteria isolated and identified from the samples showed to be Acinetobacter species and Pseudomonas species. Pseudomonads are one of the most group of important organisms found in MWF [4]. The PASCO ID Tri-Panels were used to identify the microorganisms, however it was not always possible match an organisms' name to the biotype number.

The results from the tests performed on Acinetobacter lwoffi showed that this organisms displayed only slight differences in the pH and the biocide tests. The ratio of the MIC/recommended efficacy level was approximately the same for the three biocides. The test in the mineral base medium showed that Acinetobacter lwoffi isolated from Location B (MWF from the south side) was able to grow in this medium whereas none of the other Acinetobacter lwoffi tested showed this growth in the 11 days.

The pH tests showed that most of the organisms isolated grew in a pH range of 6 to 8 while some grew at pH 9. The pH tests dealing with bioreactor sand showed that if the microorganisms were left in natural environment, they could survive in an environment with a much lower pH. One microorganism isolated from the sand at pH 4.5 was to able to grow at pH 4.5. This microorganism was not identified at the time.

The results of the biocide tests on other bacteria demonstrated the variance that one would have liked to see with Acinetobacter lwoffi. It must be remembered that these bacteria were all different and from different locations. The data from these bacteria were not as complete as the ones obtained from the Acinetobacter lwoffi thus no conclusion could be drawn. If they had been isolated each location than one would have had data to compare the results. Based on the MIC/recommended efficacy dose, one could say that the bacteria in Table 17 can tolerate a higher level of "Biocide B" than "Biocide C" as indicated in Tables 18 and 19. Thus indicating that Biocide C might be a stronger inhibitor than Biocide B.

The mineral base medium test indicated that some microorganisms could grow in this medium while other could not. The growth of the bacteria in this medium was not depent upon the location the it was isolated from. The results indicated that it was based upon the type of microorganisms. After three weeks in the mineral base medium and "Biocide B", there was no apparent growth in any of the flasks. To determine if the bacteria survived, plates counts were done on them (Table 21). The results from these counts showed that the bacteria had a harder time surviving in this environment than the two environment separately. This environment might be to stressful for the organisms, even though they did survive in low numbers, to reproduce.

PASCO MIC PANELS, MIC/ID PANELS, BREAKPOINT/ID PANEL and ID TRI-PANEL for use in ANTIMICROBIAL SUSCEPTIBILITY TESTING and IDENTIFICATION of GRAM-NEGATIVE MICROORGANISMS

INTENDED USE

PASCO MIC PANELS are used to quantitatively measure the in vitro susceptibility of microorganisms to a battery of antimicrobial agents. The principle of the classical broth dilution method is utilized to determine the minimum inhibitory concentration (MIC) of each antimicrobial agent that will inhibit visible growth of an infectious organism in vitro.[3,10,11,22,30,31] The procedures described herein are based on current recommendations of the National Committee for Clinical Laboratory Standards (NCCLS).[25]

The MIC GRAM-NEGATIVE PANEL contains antimicrobial concentrations used primarily to test gram-negative organisms, whereas those contained in the MIC GRAM-POSITIVE PANEL are generally used to test gram-positive organisms. The RESISTANT'S PANEL is used to test either gram-positive or gram-negative organisms with antimicrobial agents of specific interest.

The PASCO Identification System utilizes an optimized test set of 30 biochemical substrates and normalized likelihood as the statistical basis for identification of the family *Enterobacteriaceae* and other aerobic gram-negative bacilli.[5,6,17,18] Three sets of biochemical substrates are used in the ID TRI-PANEL to allow identification of three organisms simultaneously, whereas the PASCO MIC/ID GRAM-NEGATIVE PANEL and BREAKPOINT/ID GRAM-NEGATIVE PANEL contain one set of biochemical substrates along with antimicrobial agents to identify and determine the antimicrobial susceptibility of gram-negative bacilli. Although the BREAKPOINT/ID GRAM-NEGATIVE PANEL fully dilutes amikacin, gentamicin and tobramycin to facilitate lower dosing of these drugs and thereby decrease the risk to the patient of nephrotoxicity and ototoxicity due to high serum levels, the majority of the antimicrobial agents are included in an abbreviated format of two and three concentrations to allow testing of additional antimicrobials. It has been indicated by NCCLS that, upon using the abbreviated format panels, it is advisable to report a qualitative result (i.e., susceptible, moderately susceptible or resistant); however, the MIC may also be reported if desired.[25]

PRINCIPLES OF THE PROCEDURE

The MIC test panels contain antimicrobial agents diluted to appropriate test concentrations for blood and soft tissue infections, as well as those agents specifically indicated for lower urinary tract infections. As recommended by NCCLS,[25] the diluent for the antimicrobial dilutions is Mueller Hinton broth supplemented with calcium and magnesium to ionized physiologic levels. Also as recommended, sodium chloride is added to the broth diluent used for the oxacillin concentrations on the Gram-Positive Panel. This hypertonic broth diluent enhances detection of most oxacillin-resistant or methicillin-resistant staphylococci (MRSA).[25]

The biochemical substrates and reagents used in the PASCO Identification System are based on conventional media formulations.[4,21,29,32] Twenty-seven of the 30 tests employed are used to identify oxidase-negative organisms and a second 27-test subset is used to identify oxidase-positive organisms. Specific performance principles for each substrate are provided below.

CARBOHYDRATE UTILIZATION: Utilization of the specific carbohydrate results in acid formation. The subsequent pH drop is detected by brom thymol blue indicator which changes from green to yellow.

INDOLE: Indole, produced from the breakdown of tryptophan by tryptophanase, reacts with para-dimethylaminobenzaldehyde (Kovacs' Reagent) to produce a pink- to red-colored complex.

TRYPTOPHAN DEAMINASE: Indole-pyruvic acid, formed by the oxidative deamination of tryptophan, produces a reddish-brown color in the presence of ferric ions.

ESCULIN: Hydrolysis of esculin results in the formation of esculetin and glucose. Esculetin combines with ferric ions to produce a brown colored complex.

VOGES-PROSKAUER: Acetoin, produced from sodium pyruvate, combines with creatine and alpha-naphthol at an alkaline pH to produce a red color.

ARGININE, LYSINE, ORNITHINE: Anaerobic catabolism of arginine, lysine and ornithine results in formation of the corresponding basic amine, which is detected by brom cresol purple indicator.

UREA: Hydrolysis of urea by the enzyme urease results in formation of ammonia. The subsequent pH rise is detected by brom thymol blue indicator which changes from green to blue.

GLUCOSE FERMENTATION: Fermentation of glucose results in production of acidic end products. The subsequent pH drop is detected by brom thymol blue indicator which changes from green to yellow.

ONPG: Hydrolysis of ortho-nitrophenyl-ß-galactopyranoside (ONPG) by beta galactosidase releases yellow ortho-nitrophenol from the colorless ONPG.

CETRIMIDE: Resistance to cetrimide is demonstrated by growth in broth supplemented with cetyltrimethyl ammonium bromide.

MALONATE, CITRATE: Utilization of citrate or malonate as a sole carbon source results in the formation of alkaline end products which change brom thymol blue indicator from green to blue.

NITRATE: Reduction of nitrate to nitrite is detected by the addition of N,N-dimethyl-1-naphthylamine and sulfanilic acid, which combine with nitrite to form a red-colored compound. Complete reduction of nitrate to ammonia or nitrogen gas may be observed in some nitrite-negative reactions and is confirmed by the addition of zinc dust which detects the presence of unreduced nitrate.

CEPHALOTHIN, COLISTIN, KANAMYCIN, PENICILLIN, TOBRAMYCIN: Resistance to specific concentrations of these antimicrobial agents is demonstrated by growth in Mueller Hinton broth containing these agents.

The panels are frozen immediately after preparation and are shipped to the customer in the convenient frozen microtiter format. They must be stored at -20°C or colder in a noncycling freezer. Test panels are thawed prior to use, inoculated with the test organism, incubated 16 to 20 hours and observed for visible growth or color changes, as described above, to determine the identification of the organism. The lowest concentration of each antimicrobial agent with no apparent visible growth of the test organism is recorded as the minimum inhibitory concentration (MIC) expressed in micrograms per milliliter (µg/ml).

REAGENTS

Refer to Table 3 for current information regarding the contents of each individual panel.

PRECAUTIONS

Panels are For In Vitro Diagnostic Use.

Observe aseptic techniques and established precautions against microbiological hazards throughout all procedures. Since inoculated panels may contain potentially pathogenic organisms, all materials should be autoclaved prior to disposal.

Precautions for each of the substrate reagents are provided on the respective reagent label.

STORAGE

Panels are delivered frozen and must be stored at -20°C or colder in a noncycling freezer. -70°C is specified for certain antimicrobial agents. (See Table 3.) Once thawed, a panel must be used within 2 hours or discarded. Repeated freezing and thawing may reduce antimicrobial potency.

PRODUCT DETERIORATION

Do not use panels that show signs of contamination, thawing during shipment, or that fail to provide proper reactions or endpoints with the quality assurance organisms discussed below. When stored as recommended, the antimicrobials and biochemical substrates will retain their potency until the stated expiration date.

SPECIMEN COLLECTION AND PREPARATION

Collect specimens and place on primary isolation media according to normal laboratory practice.[14,17,19] When using the Gram-Negative Identification System, confirm that the isolate is gram-negative and determine its oxidase reaction before proceeding.

PROCEDURE

Materials Provided

Frozen panels

Materials Required but not Provided

Brain Heart Infusion Broth, 0.5 ml
Diluent with Tween 80, 12.5 ml tube
MIC Blood Supplement, 12.5 ml tube
Fildes w/MGH w/o PABA, 12.5 ml tube
Plated culture media
Mineral oil, heavy
Oxidase reagent (Kovacs')
Indole reagent (Kovacs')
10-12% ferric chloride (TDA Test)
40% potassium hydroxide (VP Test)
5% alpha naphthol (VP Test)
0.8% sulfanilic acid (Nitrate Test)
0.6% N,N-dimethyl-1-naphthylamine (Nitrate Test)
Zinc dust (Nitrate Test)
ElectraCode™
Pasco Biotype Codebook
Worksheet to record patient results
MIC/ID Quality Assurance Worksheet to record assurance organism results
Inoculating loops, 0.001 ml
Freezer, -20°C or lower (noncycling)
Freezer, -70°C when specified
Incubation strips
Incubator, 35±1°C (humidified, non-CO_2)

Pasco View Box
Pasco Cover Trays (for use as lids)
Pasco Disposable Inoculator Set for MIC and MIC/ID panels
Pasco Disposable Inoculator Set for ID TRI-PANELS
Vortex mixer
Bacti-cinerator or Bunsen burner
25 µl pipette or pipettor with sterile tips
Spectrophotometer (for turbidity standard technique)
McFarland standard #0.5 (for turbidity standard technique)
Patient MIC Report Form
Quality assurance organisms
Acinetobacter lwoffi ATCC® 19002
Enterobacter cloacae ATCC® 43091
Escherichia coli ATCC® 25922
Escherichia coli ATCC® 35218
Klebsiella oxytoca ATCC® 43086
Proteus mirabilis ATCC® 7002
Pseudomonas aeruginosa ATCC® 27853
Pseudomonas aeruginosa ATCC® 43088
Staphylococcus aureus ATCC® 29213
Streptococcus faecalis ATCC® 29212

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PERFORMING MIC AND ID TESTING

Thawing trays. Remove needed panels from freezer storage. Remove excess air from the bag of remaining panels, promptly reseal and return to the freezer. Place a clean, empty cover tray on top of each panel and allow the panels to equilibrate to room temperature before inoculating (approximately 30 minutes). Do not allow the panels to remain at room temperature longer than 2 hours before inoculation.

Inoculum preparation: The recommended procedures for inoculum standardization are the Stationary Phase Technique and the Turbidity Standard Technique.

Stationary Phase Technique

The bacterial suspension is allowed to grow to its stationary phase, followed by a simple one-step dilution to the desired concentration, as described by Barry et al.[2] Select growth from 5-10 isolated colonies on primary isolation agar plate and prepare a slightly turbid suspension in 0.5 ml of brain heart infusion (BHI) broth and incubate at 35±1°C for 4-6 hours. This will result in approximately 10^9 CFU/ml. Pipet 25 µl of this stationary phase suspension into a screw capped tube containing 12.5 ml Diluent w/Tween 80. Upon final dilution in the panel, the bacterial suspension will be approximately 10^5 CFU/ml.

Turbidity Standard Technique

Although the inoculum for most organisms can be consistently standardized by the stationary phase method, a more standardized inoculum can be achieved for slower-growing organisms by adjusting the turbidity of a suspension of the organism to that of a McFarland standard #0.5.[33] The suspension can be prepared from a broth culture; however, current NCCLS recommendations state that resistant staphylococci can be better detected when the inoculum is prepared from an agar plate.[25] This direct inoculum technique allows recovery of a more representative number of the slower-growing oxacillin-resistant strains that are present in small numbers in a heterogenous culture along with oxacillin-susceptible strains. Perform this procedure as follows.

Prepare the inoculum from growth of a 24-hour culture on agar. Transfer organisms from the agar plate to BHI broth to yield a turbidity equivalent to a McFarland standard #0.5 when using spectrophotometry, nephelometry or visual comparison. This is approximately 10^8 CFU/ml. Pipet 0.25 ml of this standardized suspension into a screw-capped tube containing 12.5 ml Diluent w/Tween 80. Invert 8-10 times to mix. Upon final dilution in the panel, the bacterial suspension will be approximately 10^5 CFU/ml. (The Prompt Inoculation System manufactured by Minnesota Mining and Manufacturing Co. (3M) has been found to be reliable in yielding reproducible direct inoculum results.[27] Consult the 3M package insert for further information.)

Fastidious Organisms

NCCLS currently recommends the Turbidity Standard Technique, described above, for preparation of the inoculum of fastidious, slower-growing organisms. The subsequent dilution of the organism suspension should be prepared in 12.5 ml of the appropriate supplemental diluent, i.e., Haemophilus-Fildes w/MGH w/o PABA (which supplies X and V growth factors) and streptococci-MIC blood supplement (lysed horse blood). Upon inoculation, described below, do not incubate in CO_2 unless necessary for growth. For an explanation of supplements for other fastidious organisms, refer to the NCCLS document.[25]

Tray inoculation: After the proper dilution is prepared, the bacterial suspension is dispensed into the inoculum tray.

1. Orient the panel so that the labeling may be read, placing the color-coded sterility wells toward the user.
2. Position the inoculator so that the missing pin will be at the lower right. This orientation assures that the negative control well will not be inoculated.
3. Remove the inoculator from the inoculum tray and gently pour the well mixed bacterial suspension into the inoculum tray, providing for dispersal throughout the channels of the tray. It may be necessary to tip the tray slightly from front to back to ensure dispersal.
4. Gently lower the inoculator tips into the channels of the inoculum tray and allow all tips to fill by capillary action. It is not necessary to apply pressure to any part of the inoculator.
5. Lift the inoculator, taking care not to touch the tips, and lower it into the microtubes of the panel. When inoculating the ID TRI-PANEL, take care to place the inoculator within the sections delineated on the panel. Overlapping or double inoculating a section will cause erroneous results and the tests will have to be repeated. Proper alignment at this step is most easily accomplished by holding the inoculator at the corners with the thumb and forefingers of each hand. The 5 µl inoculum will transfer to the wells by capillary action. The 1:20 final dilution of the inoculum suspension results in a microtube concentration of approximately 10^5 CFU/ml.
6. Return the inoculator to the inoculum tray and dispose of both, along with other contaminated materials.

Purity and colony count subculturing. Using a 0.001 ml (1 µl) loop, mix the contents of the positive growth-control well and subculture from it onto a suitable plated medium. The resulting colony count should be approximately 100 colonies. If a mixed culture is detected, the MIC and ID test results are invalid.

Biochemical overlays. Using a dropper bottle, overlay the OFG, ARG, LYS, ORN and URE wells with 3 drops of heavy weight mineral oil. These wells are underlined on the panel.

Incubation strip. Place an incubation strip over the biochemical portion of the panel, taking care not to cover the adjacent antimicrobial wells.

Incubation. Place a clean, empty cover tray on each inoculated panel and incubate under humidified conditions (70-80%) at 35±1°C for 16-20 hours in a non-CO_2 incubator (unless CO_2 is required for the growth of a fastidious organism). If it is necessary to stack the panels, do so only in groups of five or less to ensure even thermal distribution. Avoid disturbing the placement of the Incubation Strip on each panel while adding a cover tray or during stacking. After removing panels from the incubator, either allow them to equilibrate to room temperature before reading or take care to remove condensation from the bottom surface of the plastic tray that might interfere with reading.

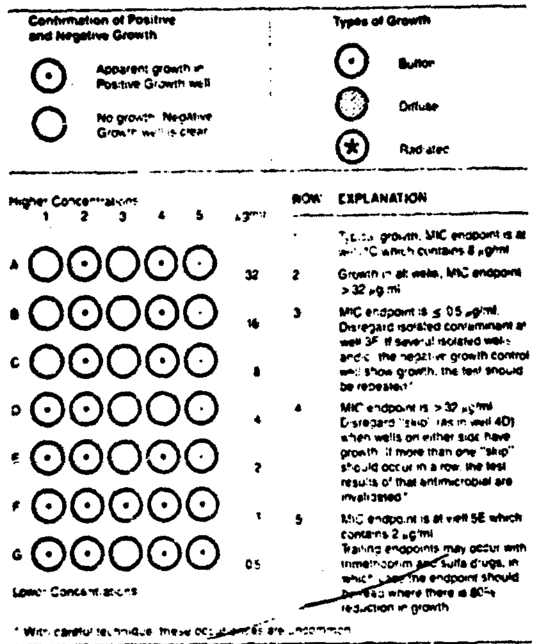
RESULTS

MIC INTERPRETATION

To determine the minimum inhibitory concentration (MIC), read the test panel against an indirectly lighted background. Due to the V shape of the well, bacterial growth in the antimicrobial solutions will usually precipitate to form a concise white button. However, some bacterial growth (particularly fastidious growers) will demonstrate turbidity or haziness throughout the antimicrobial solution that should be interpreted as growth. Observe the positive growth control to determine the growth typical of the test organism.

1. Examine the positive and negative growth control wells. If the negative control well shows no evidence of growth and if there is visible growth in the positive growth control well, the endpoints for the antimicrobials can be read.
2. "No growth" in the antimicrobial solutions is recorded when there is no turbidity or a white precipitate in the well.
3. The MIC is recorded as the lowest concentration of antimicrobial solution showing complete inhibition of growth (clear). When reading from the least concentrated dilution to the most concentrated, the MIC is the lowest concentration in which no growth appears. If all dilutions for an antimicrobial demonstrate growth, the endpoint is recorded as "greater than" the highest concentration tested. If all dilutions for an antimicrobial show inhibition of growth, the endpoint for that antimicrobial is recorded as "less than or equal to" the lowest concentration tested.
4. If there is a clear well in a series of growth wells, i.e., growth at 2 and 8 mcg/ml but not at 4 mcg/ml, the skip should be ignored and the MIC recorded as 16 mcg/ml. (See Figure 1.)* If spot contamination is suspected, as indicated by growth in the negative growth control well or by a pattern of isolated growth wells, the test should be repeated for all antimicrobials.
5. The antimicrobial action of such drugs as trimethoprim and the sulfas permits growth of a few generations of the organism before inhibition is complete. As a result, a small button of growth may be observed, in which case the endpoint may be interpreted to be the concentration in which there is an 80% reduction in growth when compared with the positive growth control well.

FIG. 1. Illustration of various test results that may occur and the interpretation of each result



* With careful technique, these occurrences are uncommon.

BIOCHEMICAL IDENTIFICATION

Read the biochemical reactions against the white background of the Pasco View Box, with the exception of Cetrimide and the antimicrobial wells, which are read against the black background. Since Cephalothin 8 µg/ml and Tobramycin 4 µg/ml are located in the MIC portion of the MIC/ID panel, but are used for identification purposes, they have been highlighted to facilitate ease of recognition and reading. Examine each biochemical substrate for color change or for the presence of growth and then proceed as follows.

Criteria for Adding Reagents

1. If the isolate is **Glucose (GLU) positive** after the initial 16-20 hour incubation, **add reagents** and read reactions as indicated below.
2. If the isolate is **Glucose (GLU) negative** after the initial 16-20 hour incubation, **but 3 or more** alternate tests excluding Colistin (CL), Penicillin (P), Kanamycin (K), Cephalothin (CF), and Tobramycin (TO) are positive, add reagents and read reactions as indicated below.
3. **If the isolate is Glucose (GLU) negative** after the initial 16-20 hour incubation **and less than 3** alternate tests are positive, replace the Incubation Strip and reincubate for an additional 24 hours before adding reagents.

Adding Reagents

1. To the Voges-Proskauer (VP) well add 1 drop of 40% potassium hydroxide followed by 1 drop of 5% alpha-naphthol. Wait 10 minutes before considering the reaction negative.
2. To the indole (IND) well add 2 drops of Kovacs' indole reagent. Read within 2 minutes after adding the reagent.
3. To the tryptophan deaminase (TDA) well add 1 drop of 10-12% ferric chloride reagent. Read within 2 minutes after adding the reagent.
4. To the nitrate (NIT) well of oxidase-positive organisms only, add 2 drops of 0.8% sulfanilic acid and 2 drops of 0.6% N,N-dimethyl-1-naphthylamine. Read within 2 minutes after adding the reagents. Confirm negative reactions by adding zinc dust. Wait 10 minutes before considering the reaction positive.

Organism Identification

Identification of gram-negative organisms may be achieved by using the Pasco Biochemical Identification Percent Chart or through the use of the Pasco Biotype Codebook or ElectraCode™, all of which are available from Pasco. All reactions included in the biochemical chart, other than supplemental tests, and all identifications were generated using Pasco's own data base. The Pasco computer-assisted identification system employs 27 oxidase-negative and 27 oxidase-positive biochemical tests. These tests are grouped into three-test subsets, shown below, which are then used to generate a nine-digit octal number for both oxidase-negative and oxidase-positive gram-negative organisms.

OXIDASE-NEGATIVE TEST SET

									VALUE
OF/G	ARA	SOR	RAF	ARG	URE	CF	IND	CET	4
GLU	TRE	MEL	RHA	LYS	CIT	ESC	TDA	CL	2
MAN	CEL	SUC	ADO	ORN	MAL	ONPG	VP	P	1

OXIDASE-POSITIVE TEST SET

									VALUE
OF/G	ARA	SOR	ARG	URE	NIT	IND	CET	K	4
GLU	TRE	MEL	LYS	CIT	ESC	TDA	CL	CF	2
MAN	CEL	SUC	ORN	MAL	ONPG	VP	P	TO	1

The biotype code number is generated by recording the positive and negative reactions and then adding the positive values in each three-test subset. Results obtained with supplemental tests are not used in the biotype number but may be required to confirm an identification. A computer-assisted identification service is available at 800-321-9813 for those organisms whose profile numbers are not found in the Pasco Biotype Codebook.

GUIDELINES TO THE USE OF MICs

1. The minimum inhibitory concentration (MIC), reported in micrograms/milliliter (µg/ml), is the

Interpretive Criteria for Biochemical Reactions

CODE	TEST	REAGENTS	POSITIVE REACTION	NEGATIVE REACTION	COMMENTS
OF/G	Glucose Fermentation	None	16 hr-Yellow to yellow-green*	Blue, green or green-yellow	
			42 hr-Bright yellow	Blue, green or green-yellow	
GLU MAN ARA TRE CEL SOR MEL SUC RAF RHA ADO	Glucose Mannitol Arabinose Trehalose Cellobiose Sorbitol Melibiose Sucrose Raffinose Rhamnose Adonitol	None	Yellow to yellow-green	Blue, green or green-yellow	For suspected Pasteurella sp. see below**
ARG	Arginine	None	Fermenters Purple	Yellow, green-gray or gray	For non-fermenters examine the Arg, Lys and Orn wells for relative differences in alkalinity. Positive reactions tend to be significantly more purple (alkaline) than the reactions obtained with other amino acids. Negative reactions tend to be yellow to light purple and comparable to each other in color.
			Non-fermenters Purple (see comments)	Yellow, gray or light purple	
LYS ORN	Lysine Ornithine	None	Fermenters Purple or light to dark gray	Yellow or green-gray	
			Non-fermenters Purple (see comments)	Yellow, gray or light purple	
URE	Urea	None	Blue to blue-green	Yellow, green or green-blue	
CIT MAL	Citrate Malonate	None	Blue to blue green	Yellow, green or green-blue	
NIT	Nitrate	2 drops of 0.8% sulfanilic acid and 2 drops of 0.6% N, N-dimethyl-1-naphthylamine	Pink to red	Colorless	Read within 2 min after addition of reagents. Confirm negative reactions by adding zinc dust
		After zinc dust	Colorless	Red	Wait 10 min before considering positive
ESC	Esculin	None	Light to dark brown	Tan or beige	
ONPG	o-Nitrophenyl ß-D-galactoside	None	Yellow	Colorless	Any evidence of yellow is positive. The Cetrimide well should be used as the negative control
IND	Indole	2 drops of Kovacs' Indole reagent	Pink to red ring	Colorless to yellow	Read within 2 min after addition of reagent
TDA	Tryptophan Deaminase	1 drop of 10-12% ferric chloride	Brown, red or orange	Yellow to yellow orange	Read within 2 min after addition of reagent
VP	Voges Proskauer	1 drop of 40% KOH followed by 1 drop of 5% alpha-naphthol	Pink to red	Colorless to yellow	Wait 10 min before considering the reaction negative. Development of a pink color after 10 min may occur with some organisms and should be considered a negative reaction
CET	Cetrimide	None	Growth	No growth	Any evidence of growth as compared to the sterility well is positive
CL P K CF TO	Colistin 4 mcg/ml Penicillin 4 mcg/ml Kanamycin 4 mcg/ml Cephalothin 8 mcg/ml Tobramycin 4 mcg/ml	None	Growth	No growth	Any evidence of growth as compared to the sterility well is positive

* For all hyphenated descriptions, the color listed first is the predominant color, i.e., yellow-green is more yellow than green.

** If the isolate demonstrates equivocal reactions with most of the carbohydrates and the glucose fermentation (OF/G) is positive, Pasteurella should be suspected. Under these conditions, carbohydrate reactions should be scored as positive on the basis of any evidence of yellow for this genus only. All other reactions should be read as described above.

lowest concentration of an antimicrobial required to inhibit or kill an infectious organism in vitro. To be effective in vivo, the concentration of the antimicrobial at the site of the infection should be greater than or at least equal to the MIC at all times.

2 Important factors to consider when determining the best antimicrobial for treatment are the achievable peak concentrations (1 hour after the dose) and the valley concentrations (immediately before the next dose) in the various body compartments.[27] For instance, some antimicrobials diffuse well into the soft tissue and are highly concentrated in the urine but only minimally penetrate the cerebral spinal fluid. MIC results attained on Pasco test panels can be interpreted using the accompanying Worksheets and computer assisted programs, according to the current NCCLS MIC Interpretive Standards of three Categories of Susceptibility described below.[25] The interpretive categories are based on antimicrobial concentrations attainable in the serum/soft tissue and, in some specific cases, concentrations attainable in the urine.

Susceptible: Infecting organism is inhibited by levels of antimicrobial agent attained in the blood or tissue on usual dosage, including oral administration when applicable. For this category, documented clinical efficacy and naturally occurring distributions of the organisms' MICs are also considered.

Moderately susceptible: Infecting organism is inhibited only by blood levels achieved with maximum dosages indicated in the drug package-insert literature. Some strains may be treatable if the infection occurs in the urinary tract where antimicrobial concentrations attained in the urine considerably exceed those attained in blood, i.e., lower urinary tract infection.

Resistant: Infecting organism is resistant to usually achievable systemic concentrations of the antimicrobial agent.

AMINOGLYCOSIDE SYNERGY SCREEN

Since penicillin or ampicillin used alone for the treatment of severe enterococcal endocarditis often results in failure, the standard therapeutic regimen has been a combination therapy of a penicillin or ampicillin plus an aminoglycoside such as streptomycin.[6,23,24,29] For enterococci, aerococci and non-enterococcal streptococci, the designation "moderately susceptible" implies the need for high dose penicillin or ampicillin for endocarditis and serious invasive tissue infections that may require (always with enterococci) combined therapy with an aminoglycoside (gentamicin) for improved therapeutic response and bactericidal action. Synergy between ampicillin or penicillin and an aminoglycoside is best predicted for enterococci by screening for susceptibility to 500 or 2000 μg/ml of gentamicin or 2000 μg/ml of streptomycin.[25]

QUALITY ASSURANCE TESTING

ROUTINE QUALITY ASSURANCE

1 A negative control well is provided on each panel. If this well exhibits growth, the test has been contaminated and must be repeated.
2 A positive growth control well is provided on each panel to demonstrate growth typical of the test organism in the test medium without antimicrobial inhibition. If there is no growth in this well, the test must be repeated.
3 As recommended by the NCCLS committee,[25] quality assurance of the antimicrobial agents contained in the panel is best done using endpoints of Reference Strains. It was previously recommended that QA testing be performed daily; however, based on proven reproducibility of endpoints and overall good performance of the microdilution method, recent recommendations by the College of American Pathologists (CAP) and others[25] indicate that weekly testing is sufficient, providing that each new lot of panels is tested.

TABLE 1 lists the recommended Reference Strains along with the acceptable endpoint ranges. If the modal endpoint is anything other than mid-range, it will be listed. A minimum of one, and in most cases 2 or 3 on-scale endpoints can be achieved for each antimicrobial when using a combination of the organisms listed. Refer to the MIC/ID Quality Assurance Report for a summary of Reference Strains recommended for each specific panel. The NCCLS Recommended Reference Strains have been used whenever possible, and the endpoints included are in agreement with NCCLS published endpoint ranges unless otherwise noted.

4 TABLE 2 lists the acceptable results for Quality Assurance organisms for each biochemical substrate.
5 Colony counts and culture purity should be checked for each isolate (outlined in PROCEDURE). Approximately 100 colonies should be recovered, assuring standardization of the inoculum. Also, if a mixed culture is detected, the test results should not be reported.
6 Quality assurance testing of the ID portion of the MIC/ID panel should be performed with each new lot of trays and on a regular basis in accordance with in-house quality assurance procedures.

MANUFACTURER QUALITY ASSURANCE

Each lot of test panels manufactured must meet quality assurance criteria for pH, freedom from interfering levels of microorganisms, and performance testing with quality assurance organisms. If the MIC endpoints for reference strains are within the ranges listed on TABLE 1 and if the biochemical reactions for organisms agree with those listed on TABLE 2, and if all other criteria are acceptable, the lot of panels is considered satisfactory for distribution. If there are any deviations from the above criteria, the lot is not distributed.

PERFORMANCE CHARACTERISTICS

Evaluations of the test panel methodology indicate that accurate and reproducible results, comparable to Reference Standard Methods, can be obtained.[?] Using the criteria of plus or minus one dilution to represent 100% agreement, test results indicate there is a 100% agreement of the Pasco System to the Reference Method for the following antimicrobials: ceftriaxone, ceftazidime, amoxicillin/clavulanic acid, ampicillin/sulbactam, [illegible], ciprofloxacin, imipenem and ticarcillin/clavulanic acid, and 98.5% agreement of test results for cefonicid.

In an internal study, 102 clinical strains were tested using the BREAKPOINT/ID GRAM-NEGATIVE PANEL and the MIC GRAM-NEGATIVE PANEL; endpoint results were compared. Where direct comparisons of MICs were possible, it was found that the MICs were identical or within the acceptable range of plus-or-minus one dilution for all antimicrobials. Additionally, 91% of the strains had identical interpretive categories (i.e., susceptible, moderately susceptible or resistant). Of the remaining 9%, no major discrepancies were noted. (A major interpretive discrepancy would be considered one in which the BREAKPOINT/ID GRAM-NEGATIVE PANEL demonstrated a "susceptible" category and the comparison demonstrated a "resistant" category.)

Inter- and intra-laboratory reproducibility as well as the potency of each antimicrobial is essentially checked and verified each time the MIC results for quality assurance are found to be equivalent to those recommended by NCCLS.[25] Deterioration of an antimicrobial (loss of potency) would result in endpoints at higher concentrations than the acceptable values listed.

In a study performed by the Centers for Disease Control (CDC), identification results attained using the PASCO Identification System were compared with those obtained using conventional biochemical and serological techniques. Of the 536 cultures of gram-negative bacilli, including typical and atypical strains of fermenters and nonfermenters, the PASCO System identified the most commonly encountered organisms at a 95% or better accuracy rate and identified the less frequently encountered enteric and nonfermenting organisms at an acceptable level. Identification of the infrequently encountered oxidase-positive fermentative organisms was quite acceptable at a nearly 80% level.

Please refer to the Pasco Biochemical Identification Percent Charts for expected results with the oxidase-negative and oxidase-positive organisms included in the data base.

LIMITATIONS OF THE PROCEDURE

MIC

1 All of the factors under GUIDELINES TO THE USE OF MICs are important in determining the drug of choice and dosage, route, and frequency of administration. However, the final interpretation of the MIC and the resulting chemotherapy must be determined by the attending physician having knowledge of the medical history and specific needs of the patient, the pharmacokinetics of the drug, and previous experience in treating the infecting organism.
2 The microdilution panel is recommended for MIC determination of rapidly growing aerobic organisms only.
3 Resistant strains of *S. aureus* produce β-lactamase and the testing of penicillin instead of ampicillin is preferred. Penicillin G should be used to test the susceptibility of all staphylococci to all penicillinase-sensitive penicillins, such as ampicillin, amoxicillin, azlocillin, bacampicillin, hetacillin, carbenicillin, mezlocillin, piperacillin and ticarcillin. Results may also be applied to phenoxymethyl penicillin or pheneticillin. Only one category of susceptibility is appropriate for penicillinase-negative staphylococci. A penicillin MIC of ≤0.03 μg/ml usually implies lack of β-lactamase production, and MICs of ≥0.25 μg/ml should be considered resistant; staphylococci with MICs between these values may or may not produce the enzyme. Laboratories should perform an induced β-lactamase test on strains with these MICs. Excerpt from NCCLS M7-A-S2.[25]
4 Use of the hypertonic broth diluent and the alternative direct method of inoculum preparation is recommended for detection of methicillin-resistant staphylococci. *S. aureus* strains exhibiting heteroresistance to one of the penicillinase-resistant penicillins (MRSA) must be reported as resistant to cephalosporins and other newer β-lactams such as amoxicillin/clavulanic acid, ampicillin/sulbactam, imipenem and ticarcillin/clavulanic acid, regardless of the in vitro test results. Methicillin-resistant, coagulase-negative *Staphylococcus* sp. also appear not to respond well to these drugs, but fewer data are available than with MRSA.[25]
5 Investigative studies of the in vitro activity of cefamandole against *Enterobacter* sp. have demonstrated a high frequency of resistant variants with broth cultures of these species, suggesting that chemotherapy with cefamandole should be evaluated carefully when compared with other cephalosporins or other antimicrobial agents.[8]
6 Please refer to M7-A-S2 for the general MIC Interpretative Standards of Three Categories of Susceptibility and note the NCCLS Recommended Interpretive Categories for each antimicrobial agent specific to certain organisms, such as *S. aureus*, *L. monocytogenes*, *B. catarrhalis*, *Haemophilus* species, etc.
7 In reference to the abbreviated antimicrobial concentration format used in the BREAKPOINT/ID GRAM-NEGATIVE PANEL, NCCLS suggests that in tests in which four or fewer consecutive concentrations are tested or in which four or more non-consecutive concentrations are tested, it is advisable to report a qualitative result (i.e., susceptible, moderately susceptible or resistant), but the MIC may also be reported, if desired.[25]

BIOCHEMICAL SUBSTANCES

1 The identification portion of the Pasco MIC/ID panels is intended for use as a diagnostic aid in the identification of aerobic, gram-negative bacilli. Since minor variation among strains of the same species may occur, strains may be encountered for which a code number is not found in the Biotype Codebook. Conversely, due to the close relatedness of various species of gram-negative bacilli, strains may be encountered for which more than one identification is possible. In both of these instances, additional biochemical testing, as well as cultural and morphological characteristics, antimicrobial susceptibility pattern, specimen source, clinical indications and serological testing may be required in order to confirm the identification of the isolate. Appropriate references should be consulted for further information.[7,12,13,16,19,34]
2 Serological typing is recommended as confirmatory testing for all presumptive *Brucella*, *Salmonella*, *Shigella* and *Vibrio cholerae* isolates.
3 Although the biochemical reactions used in the MIC/ID panels are based on conventional media, a complete correlation for all substrates does not exist. Insufficient or prolonged incubation may result in false negative or false positive results.
4 Correct interpretation of the biochemical reactions requires experience with the Pasco MIC/ID system. Reactions should be read under the specified lighting conditions using the Pasco View Box.
5 Strains from some of the more fastidious or slow-growing genera may be encountered which grow poorly in some of the biochemical substrates. These reactions are taken into account in the reaction percentages for these organisms.
6 Heavy weight mineral oil is recommended for use as the biochemical overlay in Pasco MIC/ID panels. Lighter weight oils have been shown to affect the sensitivity of some substrate reactions and should not be used.
7 To ensure proper reactions, correct placement of the Incubation Strip is extremely important. This is especially significant when stacking panels for incubation.
8 To ensure proper VP reactions, reagents should be added in the order specified. False-negative reactions may occur with some species if the alpha naphthol is added first.
9 Visible growth may not be observed in all substrates with the halophilic vibrios. These reactions are considered negative responses and are taken into account in the identification of this group of organisms.

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ABSTRACT

CHARACTERIZATION OF BACTERIA FOUND IN METAL-WORKING FLUIDS AND THE WASTE TREATMENT SYSTEM INVOLVED IN DEGRADATION OF WASTE WATER

by

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May, 1991

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Major: Biological Sciences

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This paper contains information concerning the microbial populations in Metal-working Fluids and the waste treatment system that degrades the waste water. The microorganisms were isolated and identified and then various chemical tests were performed on them to determine the effects it had the microorganisms. These tests included changing the pH of the environment, determining the biocide MIC, and changing the carbon source in the environment. These results were then analysized to see if microorganisms of the same species but from different location showed any resistant to the tests. Based on the results obtained on Acinetobacter lwoffi, the location of this bacterium did not influence the results.

AUTOBIOGRAPHIC STATEMENT

Currently, I am a Chemical Officer (Captain) in the United States Army. I was commissioned into the Army on May 1, 1985. I was selected to attend Wayne State University for advanced education in my career field. This selection is based on previous performance in all military assignments, undergraduate degree and grades. I obtained Bachelor of Science from Northern Michigan University, where I studied Biochemistry.

My assignments in the military are outlined below. I attended the chemical officer basic course from June 1985 to October 1985 at Fort McClellan, Al. From here I did a three year tour in Stuttgart, Germany. While in Germany, I was assigned as a platoon leader to the 11th Chemical Company, an assistant chemical officer to the 2d Support Command, and as the officer in charge of the 242d Chemical Detachment. I was awarded the Army Commendation Medal when I departed Germany. My next duty station was back at Fort McClellan for the officer advance course, where I was notified that I was selected to attend graduate school under the fully funded advanced civilian schooling program. Upon completion of my studies at WSU, I will go back to the Army to put this training to use.